Polar Body Genome Transfer for Preventing the Transmission of Inherited Mitochondrial Diseases

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SUMMARY

Inherited mtDNA diseases transmit maternally and cause severe phenotypes. Currently, there is no effective therapy or genetic screens for these diseases; however, nuclear genome transfer between patients’ and healthy eggs to replace mutant mtDNAs holds promises. Considering that a polar body contains few mitochondria and shares the same genomic material as an oocyte, we perform polar body transfer to prevent the transmission of mtDNA variants. We compare the effects of different types of germline genome transfer, including spindle-chromosome transfer, pronuclear transfer, and first and second polar body transfer, in mice. Reconstructed embryos support normal fertilization and produce live offspring. Importantly, genetic analysis confirms that the F1 generation from polar body transfer possesses minimal donor mtDNA carryover compared to the F1 generation from other procedures. Moreover, the mtDNA genotype remains stable in F2 progeny after polar body transfer. Our preclinical model demonstrates polar body transfer has great potential to prevent inherited mtDNA diseases.

INTRODUCTION

A common condition caused by maternally inherited mitochondrial diseases is heteroplasmy, which is a mixture of mutated and wild-type mtDNA inside the cell. Clinical phenotypes are generally related to the heteroplasmy level, i.e., the proportion of pathogenic mtDNA mutations in affected tissues. In humans, if the mutation load is over a threshold (60% in most cases), individuals may develop severe systemic diseases such as neuropathy, liver failure, myopathy, cardiomyopathy, and sensory deficit (Brown et al., 2006; Schon et al., 2012).

The frequency of pathogenic mtDNA mutations has been estimated at 1 in 200 in the general population, whereas the prevalence of mtDNA diseases is at least 1 in 10,000 individuals (Chinnery et al., 2012). Currently, inherited mitochondrial diseases are incurable and the treatments available are predominantly supportive. Genetic counseling, along with preimplantation genetic diagnosis (PGD) may not be useful for detecting the extent of mtDNA heteroplasmy (Bredenoord et al., 2008; Steffann et al., 2006), owing to certain aspects of mitochondrial genetics such as maternal inheritance (Giles et al., 1980; Hutchison et al., 1974), “bottleneck” segregation (Cao et al., 2007; Cree et al., 2008; Wai et al., 2008), and selective replication (Fan et al., 2008; Meirelles and Smith, 1997; Stewart et al., 2008). Therefore, new approaches are necessary to prevent the transmission of inherited mtDNA variants from mother to child.

With the increasing interest in developing new strategies to prevent mtDNA diseases, the UK Human Fertilization and Embryology Authority (HFEA) and the US Food and Drug Administration (FDA) have been evaluating new research approaches such as mitochondrial replacement (MR) between oocytes to treat patients (FDA, 2014; HFEA, 2012). MR therapy involves transferring the nuclear genome from a patient’s egg into an enucleated healthy egg with normal mtDNA and has been facilitated by a series of advances. Early studies involving pronuclear transfer (PNT) between mouse zygotes showed that the mtDNA-related phenotypes were corrected in a mouse model (Sato et al., 2005). In humans, PNT between zygotes has shown minor donor mtDNA carryover in early embryos (Craven et al., 2010). However, in the same PNT mouse study, PNT-generated mice, though free of mtDNA-related phenotypes, still had 5%–44% heteroplasmy level at day 300 after birth (Sato et al., 2005), largely because abundant mtDNA molecules amplify and aggregate after fertilization and contaminate the transplanted pronuclei with cytoplasm leading to carryover (Cao et al., 2007; Santos et al., 2006). In addition, PNT requires fertilizing both the donor and the recipient oocytes, which results in discarding half of
the embryos during manipulation. Recent experiments have used spindle-chromosome transfer (ST) between mature oocytes to eliminate inherited mtDNA variants in nonhuman pri-
mate and early human embryos (Paull et al., 2013; Tachibana et al., 2009, 2013). The results showed minimal carryover in pri-
mate offspring and human embryonic stem cells. However,
because the spindle apparatus is sensitive (Kitajima et al.,
2011), the outcome of spindle manipulation is operator depen-
dent. Moreover, previous reports on in vitro mtDNA analysis
of PNT- and ST-treated human cells may not accurately reflect
the in vivo mtDNA inheritance and segregation landscape in
live offspring.

Based on these considerations, we hypothesized that the first
and second polar bodies (PB1 and PB2) could be efficient and
feasible donor germ cells for the complete replacement of
mtDNA genotype (Figures 1A and 1B). PB1 and PB2 are small
germ cells that divide by meiosis. The mammalian primary
oocyte extrudes a diploid PB1 before ovulation. After fertilization
with a sperm cell, the oocyte emits a haploid PB2 and the re-
main ing fertilized zygote contains a haploid female pronucleus
(FPN) and male pronucleus (MPN). Increasing evidence in recent
decades shows that PB1 and PB2 include the same genetic
material and developmental potential as their counterparts
inside the ooplasm (Bieber et al., 1981; Hino et al., 2013;
Wakayama and Yanagimachi, 1998; Wakayama et al., 2007,
1997). Moreover, a recent study using MALBAC-based single-
cell technology showed that PB1 and PB2 faithfully possess
the same genome settings as the sister nuclei of oocyte (Hou
et al., 2013).

Using polar bodies (PBs) provides certain advantages. First,
due to oocyte-biased inheritance of mitochondria and the rela-
tively small size of PBs, PB1 and PB2 contain very few cellular
organelles, such as mitochondria, and are composed almost entirely of chromatin (Dalton and Carroll, 2013; Piko and Taylor, 1987). Thus, a minimal carryover of donor (patient) mtDNA genotype is expected in the reconstituted embryos and offspring produced by PB transfer. Second, enveloped with a cellular membrane, PB1 and PB2 can be easily visualized and handled in a micropipette without chromosome loss (Maro et al., 1986). Third, if the transfer of PBs and their sister nuclei, spindle or pronuclear nuclear, could be used together in each individual donor egg, this process would reduce by half the number of donor oocytes used and greatly increase the efficiency of MR.

Here, we report that PB1 and PB2 can be used as donor genomes to replace the genome of recipient eggs as an efficient approach of MR in a mouse model. We present a comparative analysis of using pronuclear, spindle, PB1 and PB2 transfer to generate mouse progeny to establish preclinical models for novel therapies (Figures 1A and 1B). We first compared mitochondrial distribution in oocytes and PBs. We then investigated the developmental outcomes and donor mtDNA carryovers in the F1 and F2 offspring after MR. We also conducted additional array-based genomic studies between human eggs and PBs to extend the possibilities of polar body genome transfer for clinical translations.

RESULTS

PBs Contain Fewer Mitochondria Than Their Sister Oocyte

For minimal carryover of mtDNA variants after MR therapy, it is crucial to isolate nuclear complexes with minimal donor mtDNA content. We hypothesized that PBs have fewer mitochondria than their counterparts, the spindle-chromosome complexes and pronuclei in oocytes and zygotes, respectively. To test that, we tested the mitochondrial content in oocytes and zygotes via immunofluorescence staining and digital quantitative PCR.

We first stained B6D2F1 (BDF1) mouse oocytes and zygotes with Hoechst and MitoTracker to visualize the nuclear DNA and mitochondria via confocal microscopy with z stacked images. As expected, PB1 contained few mitochondria and almost no visible mitochondria in some cases (Figure 2A). The spindle-chromosome complex was surrounded by amounts of mitochondria in some oocytes (Figure 2A). Corroborating recent findings, mitochondria aggregated around the spindle during oocyte maturation and were then distributed universally throughout the MII oocyte (Dalton and Carroll, 2013). We explain this behavior through the notion that the spindle-chromosome complex requires ATP to reassemble, migrate, and divide during meiosis, which resulted in the surrounding mitochondria aggregation. Nuclear DNA staining showed that PB1 and oocyte share similar chromosome morphologies (Figure 2A). Next, we detected the mitochondrial distribution in zygotes. PB2 contained few mitochondria, whereas in zygotes, mitochondria were abundant and distributed homogeneously (Figure 2B). This finding correlated with previous reports, wherein zygotic activation induced mtDNA aggregation (Cao et al., 2007; Santos et al., 2006). Additionally, DNA staining exhibited the same pronucleus morphology in PB2 (Figure 2B).

Next, through higher magnification of isolated karyoplasts from PB1, spindle-chromosome complex, PB2 and pronuclei, PBs were confirmed to contain fewer mitochondrial molecules than their counterparts (Figures 2C and S1A available online). The fluorescence intensity of PBs was significantly lower than that of the spindle-chromosome complex or the pronuclei (p < 0.0001, n = 10 per group) (Figures 2D and S1A). Although it is technically feasible to reduce mitochondria content by isolating karyoplasts with less cytoplasm, the chromosome might be lost because the spindle-chromosome complex does not include a nuclear membrane. In contrast, enveloped with a cellular membrane, PB1s were easier to isolate without chromosome loss.

To further investigate the mitochondrial content, we employed chip-based digital PCR to estimate the mtDNA copy number in germline karyoplasts (Figures 2E and S1B). Based on PCR amplification of single-template molecules, digital PCR is a precise analytical technique for quantifying nucleic acid samples without a standard curve for reference, and the number of positive and negative PCR reactions was used to count the number of target mtDNA molecules (Sanders et al., 2011; Taylor et al., 2014; Vogelstein and Kinzler, 1999). In general, PB1 contained 359 molecules on average (n = 80), which was significantly lower than the spindle complex (2,318, n = 32) (p < 0.0001) (Figures 2E, S1B, and S1C). We also detected significantly fewer mtDNA molecules in PB2 (1,092 on average, n = 70), than in the pronuclei (34,392 on average, n = 39) (p < 0.0001) (Figures 2E, S1B, and S1C).

Taken together, these results suggest a biased mitochondrial inheritance during meiosis, resulting in the majority of mitochondria being retained in the oocyte, and few mitochondria being extruded to the PBs. Hence, we expect that genome transfer from PB1 and PB2 can be achieved with minimal mitochondrial DNA carryover from the donor.

Mitochondrial Replacement via PB1 Genome Transfer and Spindle-Chromosome Transfer

We examined the feasibility and efficiency of PB1 genome transfer (PB1T) for replacing the mitochondrial genotype in mouse oocytes with a different mtDNA origin. The mtDNA of NZW/LacJ and BDF1 mice comes from normal inbred mice but differs by three nucleotides (Table S1). We hypothesized that each donor oocyte offered two nuclear genome sources for mitochondrial replacement (MR): PB1 and the spindle-chromosome complex. We conducted a PB1T and then a ST between oocytes.

Prior to nuclear transfer, we detected the cytoskeleton and nuclear integrity of PB1. BDF1 oocytes with live PB1 were fixed and analyzed by labeling with anti-α-tubulin antibody. PB1 contained intact microtubules that were morphologically similar to the spindle-chromosome complex of mature oocytes (13–16 hr after human chorionic gonadotropin (hCG) injection) (Figures 3A and S2A). Costaining with propidium iodide (PI) showed that chromosomes remained attached to PB1 microtubules, and labeling with the DNA damage markers of phospho-p53 and phospho-H2A.X showed no immunoreactivity, which demonstrated the nuclear integrity of PB1 (Figures 3B, S2A, and S2B). Furthermore, to address the concerns over “epigenetic” differences between PB1 and oocyte, we performed
immunostaining with antibodies to 5-methylcytosine (5mC), histone H3 tri-methyl K9 (H3K9me3), and histone H3 phospho S10 (phospho H3) between oocytes and PB1s was performed (Cantone and Fisher, 2013; Gu et al., 2010; Kota and Feil, 2010; Santos et al., 2005). PB1 exhibited very similar expression of 5mc, H3K9me3, and phospho H3 to the oocyte chromosomes (Figures 3C, 3D, and 3E). These imply that PB1 didn’t contain significant “epigenetic” alterations compared with the oocyte chromosomes, which favors the programming of PB1 genome when transferred into recipient ooplasm.

We then performed PB1T and ST in oocytes to exchange the mitochondrial genotypes. By optimizing the superovulation timing, we collected mature NZW oocytes with a live PB1 as the donor genome, which allowed us to isolate the spindle-chromosome complex and PB1 from a single oocyte. Live PB1s, with an intact plasma membrane, were easily visualized and isolated...
in a micropipette (Figure 3F and Movie S1). In total, 25 (92.6%) live PB1s were retrieved from 27 oocytes (13–16 hr post-hCG), and then transplanted into the enucleated BDF1 oocytes (Figure 3F, Table S2, and Movie S1). Immediately following the PB1 transfer, the donor oocytes were used for ST. Twenty-seven spindle-chromosome complexes were collected and subsequently fused to enucleated recipient BDF1 oocytes using HVJ-E (inactivated Hemagglutinating Virus of Japan envelope) protein (Figure 3G, Table S2, and Movie S2). The survival rates after nuclear transfer were very high in both groups; 19 (76.0%) out of 25 and 24 (88.9%) out of 27 reconstructed oocytes were collected after PB1T and ST, respectively (Table S2).

We concluded that a single oocyte could offer two donor genome sources for an efficient MR procedure. Nuclear genome transfer from 27 donor oocytes yielded 43 mitochondria-replaced oocytes, which almost doubled the MR success rates. We demonstrated that, assuming MR therapy was used in clinical settings for women that carried mutant mtDNA, PB1T, together with ST would reduce the necessary donor (patient) oocytes by one-half needed and enhance the efficiency.

Figure 3. PB1 Integrity and Mitochondrial Replacement by PB1 Genome and Spindle-Chromosome Transfer
(A) Confocal analysis of α-tubulin in PB1 and spindle-chromosome complex.
(B) Oocytes were immunofluorescently labeled with antibodies to α-tubulin and PI to visualize microtubules and chromosomes in PB1 and spindle-chromosome complex. Right image is high-magnification view of boxed area in the left.
(C–E) Representative confocal images of oocytes with PB1 costained with antibodies to (green) 5mc (C), H3K9me3 (D), phospho-H3 (E) and PI (red).
(F–G) PB1 was isolated from donor oocyte and then transferred into recipient oocyte to exchange mitochondria genotypes (F). After PB1 transfer, spindle-chromosome complex was isolated from donor and transferred to recipient oocyte (G). Experimental schemes (left) and micromanipulation images (right) are shown.
Scale bar, 10 μm. See also Figure S2 and Movies S1 and S2.
Mitochondrial Replacement via PB2 Genome Transfer and Pronuclear Transfer

We next investigated the potential for using second polar body transfer (PB2T), together with pronuclear transfer (PNT) as mitochondrial replacement (MR) approaches.

Initially, we detected the nuclear membrane integrity of PB2 and its sister FPN by costaining with antibody to Lamin B1 and PI (Goldberg et al., 2008; Houliston et al., 1988; Link et al., 2013). PB2 contained an almost identical envelope and pronucleus morphology to FPN (Figures 4A and S3). Immunostaining with phospho-p53 and phospho-H2A.X showed no DNA damage in PB2 (Figure S2B). We also colabeled Lamin B1 and PI to visualize the membrane morphology and pronucleus sizes at different pronuclear stages (PN stage, PN0-PN4) (Figure S3). At PN0-PN2, the size and lamin expression of PB2 nucleus were similar to those of the FPN (Figure S3). After the PN3 stage, the FPN was significantly larger than PB2 (Figure S3). To synchronize the nucleus size of the donor and the recipient, we reasoned that the recipient zygote at PN0-PN2 would be more appropriate for PB2 programming (Figure S3).

We then compared the “epigenetic” status, DNA methylation, and histone modification status of the PB2 genome and its sister FPN by labeling zygotes with anti-5mc, anti-H3K9me3, and anti-acetyl-H3 antibodies (Figures 4B, 4C, and 4D)(Cantone and Fisher, 2013). We observed 5-mc and H3K9me3 in the FPN but not in the MPN (Figures 4B and 4C), which indicated that the maternal genome has a protective mechanism against demethylation (Cantone and Fisher, 2013; van der Heijden et al., 2005; Liu et al., 2004; Santos et al., 2005). The fluorescent signals for the 5mc, H3K9me3, and acetyl-H3 were clear in PB2,

Figure 4. Integrity of PB2 and Mitochondrial Replacement by PB2 and Pronuclear Transfer
(A) Zygotes were immunofluorescently labeled with antibodies to lamin B1 and PI to visualize nuclear membrane and chromosomes in PB2 and pronuclei.
(B–D) Representative confocal images of zygotes with PB2 costained with antibodies to (green) 5mc (B), H3K9me3 (C), acetyl-H3 (D) and PI (red).
(E and F) PB2 was isolated from donor zygote and transplanted to female pronucleus (FPN)-enucleated recipient (E). Then pronuclei were isolated from donor zygote and transferred to enucleated recipient zygote (F). Experimental schemes are shown on left and micromanipulation images are shown on the right.
Scale bar, 10 μm. See also Figure S3, Movies S3 and S4.
which suggests that it shares similar chromatin pattern to the FPN (Figures 4B, 4C, and 4D). The similar “epigenetic” patterns in PB2 and the FPN indicate that PB2 could be used as a substitute for the FPN.

We then conducted PB2 and pronuclei transfers between the donor and the recipient zygotes. After collecting the donor zygotes, PB2 was isolated in a micropipette and then transferred into a half-enucleated recipient zygote at the PN0–PN2 stage (Figure 4E and Movie S3). The FPN of the recipient zygote was enucleated for PB2T, and the MPN remained (Movie S3). The haploid set of female genomes inside PB2 was transplanted into FPN-enucleated recipient zygotes (Figure 4E and Movie S3). In total, 30 PB2s (retrieved from 30 zygotes) was transferred into the recipients, and 28 (93.3%) survived the manipulation (Table S2). Next, 19 pronuclei karyoplasts (retrieved from 30 zygotes) from donor zygotes were transferred to enucleated recipient zygotes (Figure 4F and Movie S4). Sixteen out of 19 (84.2%) PNT-generated reconstructed zygotes survived after manipulation (Table S2). These results demonstrated that PB2 can be readily manipulated and used for MR treatment in an efficient way.

Developmental Potential after Mitochondrial Replacement

To further examine the efficiency of mitochondrial replacement treatment by different germline genome (PB1, spindle, PB2, and pronuclear) transfer techniques, we compared the developmental competence of reconstituted embryos both in vitro and in vivo.

We introduced sperm cells into reconstructed oocytes after PB1T and ST via in vitro fertilization (IVF), and the fertilization rates were calculated for both groups: 17 out of 19 (89.5%) fertilized PB1T-generated oocytes and 21 out of 24 (87.5%) fertilized ST-oocytes were obtained. The PB1T-generated embryos cleaved at a 94% frequency, and 87.5% developed into blastocysts (Figures 5A, 5B, S4, and Table S2). The results
were similar for ST-generated embryos; 100% of ST-oocytes cleaved and 85.7% developed to blastocysts (Figures 5A, 5B, and S4, and Table S2). These indicate that PB1 maintains a similar developmental potential to its sister spindle-chromosome complex. The results were comparable with the intact oocyte IVF group (p > 0.05). For PB2T and PNT, the cleavage rates were similar (96.4% and 100%, respectively), whereas the PNT-embryos yielded higher blastocyst rates (55.5% and 81.3%, respectively). Next, we tested in vivo developmental competence by transferring these MR-treated blastocysts into pseudopregnant mothers. Six living PB1T-generated F1 pups (42.8%) out of 14 transferred blastocysts were delivered from two recipients, which is similar to the ST-generated embryos (8 living pups [44.4%]) out of 18 blastocysts from 3 recipients) (p > 0.05) (Figures 5B and 5C, and Table S2). In the PB2T and PNT groups, 6 (40%) and 7 (53.8%) living F1 pups were delivered out of 15 and 13 blastocysts, respectively (Figures 5B and 5C, and Table S2). All living pups were born healthy and resired normally. The birth rates of MR-newborns were comparable to those of the intact control group (p > 0.05) (Figure 5B and Table S2). These results demonstrate that MR therapy, including PB transfer, did not compromise the developmental competence and produced living progeny from each of the four nuclear transfer techniques. In addition, the birth weights and placenta weights of all MR-generated pups were in the normal range in a manner reminiscent of the intact group (p > 0.05), which indicates that the MR treatment did not produce overgrowth phenotype (Figure 5D).

Overall, these results demonstrated that the reconstructed embryos produced using PB1 and PB2 were suitable for onward development at rates similar to the ST- and PNT-generated embryos as well as the controls.

mtDNA Carryover Analysis through Pyrosequencing

To determine the mtDNA carryover in mice delivered after MR treatment, we adopted the high-throughput pyrosequencing method, which has a detection threshold of approximately 1%, 100% specificity, and 100% sensitivity, to examine the level of heteroplasmy (Blakely et al., 2013; White et al., 2005). We exploited the C9461T SNP variant in the ND3 gene between the donor and the recipient to distinguish the mtDNA origins of MR-infants (Figures 6A and 6B and Table S1). Thus, where heteroplasmy is present, the proportion of genome donor’s mtDNA would be detected.

We first quantified the heteroplasmy proportions in the tail tips and important organs of the F1 generation infants after birth. The male infants were sacrificed to detect the levels of mtDNA variants in the important organs, namely the brain, heart, lungs, liver, and kidneys. The female infants were allocated to foster mothers and their tail tips were used to monitor mtDNA heteroplasmy. We compared the level of heteroplasmy for the four MR-treatment groups by collecting the data on the proportion of donor mtDNA in the tail tissues of fostering infants and brain tissues of sacrificed infants. The mean heteroplasmy level for all (n = 6) PB1T-generated infants was undetectable (Figures 6C and S5). The mean heteroplasmy level for the ST-generated infants was at 5.5% ± 1.4% (mean ± SD, n = 8), which is significantly higher than the PB1T-generated group (p = 0.0007) (Figures 6C and S5). The PB2T infants had 1.7% ± 2.8% (n = 6) mtDNA carryover on average, whereas the PNT infants had high level of 23.7% ± 11.1% on average (n = 7), which is significantly higher than the PB2T group (p = 0.0012) (Figures 6C and S5). These results are consistent with the findings that PBs contain far fewer mtDNA molecules than do spindle and pronuclei karyoplasts.

We surveyed the mtDNA variant levels in important organs and tissues (brain, heart, lungs, liver, and kidneys) in the F1 generation mice, which require more energy (Spreadsheet S1). As expected, the PB1T-generated infants exhibited undetectable carryover (0%) in all tissues (n = 15) (Figures 6E and S5). This confirms that PB1T can reduce the transmission of donor mtDNA to the lowest level. The level of mtDNA heteroplasmy in all biopsied tissues from ST-generated infants was detected at a medium to low level (range 0%–6.88%, n = 20) (Figures 6F and S5). The PNT-generated infants had significantly high levels of heteroplasmy; all biopsied tissues included detectable levels of mtDNA variants (range 5.55%–39.8%, n = 15) (Figures 6H and S5). In contrast, the levels mtDNA variants were undetectable in all biopsied tissues (n = 10) of two PB2T-generated infants (Figures 6G and S5). Only one PB2T-treated infant exhibited a detectable level of heteroplasmy (range 3.08%–3.62%, n = 5) (Figures 6G and S5). In addition, the heteroplasmy level between the organs investigated was consistent; thus, if mtDNA carryover was detected in one mouse, the variations were consistent between the important organs investigated.

In a previous study of ST-treated primates, a significant increase (14.2% and 16.1%) of donor mtDNA carryover was detected in biopsied oocytes from the pups’ ovaries, indicating that heteroplasmic mtDNA may undergo selective amplification during germline development, and transmit it to the next generation of offspring (Tachibana et al., 2009). To address this concern, we mated female F1 generation progeny with male C57 mice and detected whether the mtDNA genotype remained stable in the next F2 generation (Figures 6D, 6I, and S5 and Spreadsheet S1). The level of heteroplasmy in the toe tips of the F2-PB1T mice (n = 16) was undetectable (0%), which is significantly lower than in the F2-ST mice (7.1% ± 6.8%, n = 28) (p < 0.0001) (Figures 6D, 6I, and S5). This indicates that PB1 genome transfer can yield undetectable levels of mtDNA heteroplasmy in pups over generations. The F2-PNT mice exhibited 22.1% ± 18.7% carryover (n = 21), whereas the F2-PB2T group contained significantly lower mtDNA variants (2.9% ± 4.3%, n = 21) (p < 0.0001)(Figures 6D, 6I, and S5). Interestingly, for females with high levels of mtDNA heteroplasmy (such as in the PNT group) the predilection to maintain mtDNA heteroplasmy remains. And for females with undetectable level of mtDNA heteroplasmy (such as in the PB1T Group), no minority mtDNA content was detected in the F2 pups. However, when the level of mtDNA heteroplasmy was around the median (approximately 10%), then the transmission of minority mtDNA becomes random.

Taken together, the results strongly support the notion that PB transfer, especially PB1T, can be efficiently used for MR therapy and produce offspring with undetectable levels of donor-derived mtDNA over generations.
Genomic Integrity of Human Polar Bodies Is Confirmed by aCGH

To extend our findings in mouse models, we performed array comparative genomic hybridization (aCGH) to detect genomic aberrations between PB1 and its counterpart, the spindle-chromosome complex, in a human MII oocyte, PB2 and the female pronucleus in a human zygote at the single-cell level. Immunos-tainting with the DNA damage marker of phospho-p53 and phospho-H2A.X showed no signal in both human PB1s and PB2s (Figures S2B). We isolated PB1 from an MII oocyte and the spindle-chromosome complex in its cytoplasm. The single-cell aCGH analysis confirmed a similar chromosome copy number

Figure 6. mtDNA Carryover after Mitochondrial Replacement

(A) mtDNA sequence variant (m.9461C > T) in ND3 gene used for pyrosequencing to detect mtDNA heteroplasmy level. (B) Sequence pyrogram for mtDNA variant (m.9461C > T) in donor (left) and recipient (right) oocytes. (C and D) Heteroplasmy quantification of pups of F1 (C) and F2 generation (D) by pyrosequencing. Both F1 and F2 PB1T-generated infants showed undetectable mtDNA carryover, significantly lower than ST group. F1 and F2 PB2T-generated pups also showed significantly fewer mtDNA carryover than PNT group. Asterisks indicated significant changes with p value presented (Mann-Whitney test). Error bar indicate SD, with the mean value and n number shown. (E-H) Heteroplasmy levels of detected tissues from F1 pups produced by PB1 transfer (PB1T, E), spindle transfer (ST, F), PB2 transfer (PB2T, G), and pronuclear transfer (PNT, H). Brain, heart, lung, liver and kidney tissues were sampled to detect the transmission of heteroplasmy level after mitochondrial replacement. (I) Heteroplasmy levels of all F2 pups in PB1T, ST, PB2T and PNT groups. See also Figure S5, Table S1, and Spreadsheet S1.
between PB1 and the spindle-chromosome complex. Both had a diploid genome and no alterations were detected (Figures 7A, 7B, S6A, and S6B). Likewise, the aCGH analysis of a zygote confirmed the fact that PB2 had the same haploid genome as female pronucleus and no meaningful genomic alterations were detected (Figures 7C, 7D, S6C, and S6D). Our findings were consistent with previous reports that the PB and oocyte share the same genomic landscape (Christopikou et al., 2013; Geraedts et al., 2011; Hou et al., 2013), hence the nuclear transfer between the PB and oocyte can be readily performed and paves the way for PB-based MR therapy in a clinical setting.

**DISCUSSION**

Our data present evidence of efficient mitochondrial genotype exchanges using polar bodies to prevent the transmission of mtDNA variants. PB1T produces undetectable level of mtDNA heteroplasmy in all offspring. The easy visualization and efficient manipulation of PB1 and live birth thereafter confirms the feasibility of PB1T as an MR treatment. The ST-generated live offspring had low to median level of donor mtDNA variants. For ST-generated mice, the level of mtDNA heteroplasmy in our experiments was greater than in previous reports on ST-generated embryonic stem cells and primate offspring (Paull et al., 2013; Tachibana et al., 2009, 2013). This discrepancy is largely because the spindle-chromosome complex didn’t have a membrane, and to prevent chromosome loss during manipulation, certain volume of cytoplasm is retained. To achieve minimal carryover, a higher-resolution microtubule birefringence should be used to visualize the spindle apparatus. It should also be noted that the human spindle complex is smaller than the mouse counterpart; thus, human ST resulted in less variant carryover. Furthermore, ST is subject to operator-dependent factors (e.g., the quantity of cytoplasm aspirated and transferred to the recipient, which was dependent on the individual embryologists). In contrast, PB isolation and transfer can be completed in a single step, which is similar to intracytoplasmic sperm injection (ICSI), a routine practice in IVF settings (Movie S1).

The average mtDNA carryover in PNT-generated offspring was the highest (23.7%), which is consistent with previous findings that PNT treatment produced heteroplasmic progeny (Meirelles and Smith, 1997; Sato et al., 2005). This is mainly because zygotic activation induces mtDNA amplification around pronuclei (Cao et al., 2007; Santos et al., 2006). In contrast, PB2T-generated offspring harbor low or undetectable donor mtDNA carryover, which indicates that PB2 can be used as a FPN substitute to achieve minimal donor mtDNA variants during MR treatment. However, the obstacle of PB2T is that, unlike PB1, PB2 only contains a haploid set of genome, thus in our experiments the recipient oocyte is half enucleated, and the MFN is remained. Assuming that this technique is applicable in the clinic, it is particularly challenging to isolate one pronucleus in human recipient oocytes, and we presume that both pronuclei should be enucleated and then sperm cell be introduced later in the reconstructed oocytes via IVF or ICSI.

The minimal mtDNA carryover after PB1T and PB2T is consistent with the quantification results for mtDNA molecules using 3D digital PCR. For human oocytes, it has been reported that PB1 harbors approximately ten molecules, as determined using a computational model of mtDNA mutation analysis (Gigarel et al., 2011). This is largely because the sizes of human PB is significantly smaller than those of mice PB, which makes the cytoplasm volume (and the mtDNA therein) of a human PB is less than that of a mouse PB. In an earlier study, 1,000 mtDNA copies on average were detected in ten human PBs (three PBs contained almost no mtDNA copies, and four PBs included less than 500 copies) (Steuerwald et al., 2000). The differences and variations between the samples would be attributed to the difference in oocyte ages, sample size, PCR accuracy, and some unknown mtDNA dynamics that lower the mtDNA copies in mutated oocytes. We believe that further careful assessments of mtDNA copies in human PBs should be approached before clinical translations.

Given that inherited mtDNA diseases are difficult to treat, their prevention is a priority. Recently, the Nuffield Council on Bioethics has endorsed the research concerning nuclear transfer to prevent inherited mtDNA diseases (Nuffield Council on Bioethics, 2012). Inherited mtDNA variant transmission is complex because of the “bottleneck” at early oogenesis (Cao et al., 2007; Cree et al., 2008; Stewart et al., 2008). And the phenotype severity is related to a threshold effect; typically if the mutation load is greater than 60%, severe symptoms and higher mortality rates will appear (Brown et al., 2006; Schon et al., 2012; Wallace and Fan, 2009). However, studies have confirmed that even a <5% level of heteroplasmy can produce severe diseases in humans (Ballana et al., 2008). Thus MR should decrease donor (patient) mtDNA carryover to the least level. Our results indicate that polar body genome transfer, especially PB1 transfer, minimized the quantity of donor (patient) mtDNA carryover and produced offspring with an undetectable level of heteroplasmy. An additional concern over PB transfer is the “epigenetic” issue; whether PBs have the same epigenetic landscape as their oocyte counterpart. Our results showed no significant decrease in developmental outcomes after PB transfer, indicating that this technique is unlikely to cause epigenetic and imprinting defects. However, single-cell-based epigenetic profiling will be necessary to further evaluative epigenetic modifications at individual loci (Lorhongpanich et al., 2013).

There is an ongoing discussion on proceeding with human clinical trials recently, and we reason it will be important to gather more evidence from preclinical studies on the feasibility, efficacy, and safety of MR therapy (Reinhardt et al., 2013). Moreover, careful assessments of the cost and benefit for patients, ethical considerations, and assisted reproduction guidelines

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**Figure 7. aCGH Analysis of Human Oocytes and Polar Bodies**

(A–D) aCGH showed that human MII oocyte and its adjoining PB1 (A and B), female pronucleus of zygote and PB2 (C and D), respectively, shared same genomic landscape. No major alterations (amplifications or losses) were detected in PBs. See also Figure S6.
are necessary before translating into a clinical setting. The data presented in this paper show that MR therapy via polar body genome transfer has the potential to prevent inherited mtDNA diseases. The comparative analysis of different types of germ cell transfer in a mouse model confirmed that PB1T, PB2T, and ST could be readily used to exchange mtDNA without resulting in significant heteroplasmy level, and PB1T yield undetectable level of heteroplasmy over generations. Thus our mouse model studies would represent a potential approach for preventing inherited mtDNA disorders.

**EXPERIMENTAL PROCEDURES**

Detailed experimental protocols are provided in the Extended Experimental Procedures.

**Mitochondrial Replacement between NZW and BDF1 Mice**

**Experiment 1: Mitochondrial Replacement by PB1 and Spindle-Chromosome Transfer**

BDF1 and NZW females were superovulated by PMSG (5 IU) and hCG (5 IU) injection at 48 hr apart. Transferring of PB1 of NZW donor oocyte and enucleation of BDF1 recipient oocyte were done in one step with a piezo-driven actuator (PrimeTech). Then donor spindle-chromosome complex was gently aspirated to a pipette, exposed to the HVJ-E (CosmoBio), and transferred to an enucleated recipient oocyte. Reconstructed oocytes were fertilized by IVF.

**Experiment 2: Mitochondrial Replacement by PB2 and Pronuclear Transfer**

PB2 of donor NZW zygotes was aspirated into injection pipette with a piezo-driven actuator (PrimeTech). After exposure to the HVJ-E, PB2 was transferred into FPN-enucleated BDF1 zygotes. Then donor zygotes were used for pronuclear transfer to exchange mitochondria genotypes similar to spindle transfer.

**Embryos Transfer and Pups Births**

After 3 days, Blastocysts were transferred to the uterus of pseudopregnant ICR females. Sixteen days after embryos transfer, cesarian section was performed to deliver pups of F1 generation. When reached puberty, F1 females were mated with C57 males and delivered pups of F2 generation.

**Mitochondrial Distribution and Whole-Oocyte Immunocytochemistry**

Mitochondria were labeled with MitoTracker (Life Technologies) in live oocytes and isolated karyoplasts with counterstained of Hoechst 33342 (Life Technologies). For immunocytochemistry, primary antibodies include antibodies to α-tubulin (Sigma), lamin B1 (Abcam), H3K9me3 (Abcam), phospho H3 (Abcam), acetyl H3 (Millipore), 5mC (Abcam), phospho-H2AX (Cell Signaling), and phospho-p53 (Cell Signaling). Then samples were incubated overnight at 4°C and then incubated with secondary antibodies. After being washed with PBS and counterstain of nucleus chromosome with Propidium Iodide (Invitrogen), cells were analyzed by confocal microscopy (Leica Microsystems).

**mtDNA Copy-Number Analysis**

mtDNA copy-number analysis by 3D digital PCR was provided in the Extended Experimental Procedures.

**Genotyping of mtDNA Heteroplasmy Level**

All F1 mice were genotyped using DNA extracted from organs and tail tips. Toe tips of F2 pups were biopsied for mtDNA analysis. The region of mice mitochondrial genome (nucleotide position, 9201–11102) was first amplified from the total genome. The SNP used for detecting heteroplasmy is m.9461C > T. Single-stranded biotinylated PCR products were prepared for sequencing by Pyrosequencing Vacuum Prep Tool (Biotage AB). Quantification of the heteroplasmy level was achieved using allele frequency quantification method and alleles were analyzed by PyroQ-AQ software to compare the status of nucleotides at the relevant position (Blakely et al., 2013; White et al., 2005).

**Statistical Analysis**

All statistical analyses were performed using the Prism 6.0 statistical analysis program (GraphPad). Paired t test was used to access fluorescence intensity. t test, chi-square test or ANOVA was used to access mtDNA copy number, embryo development, and pups weight. Mann-Whitney test was used to access mtDNA heteroplasmy. The significance was set at p < 0.05 (‘’ denotes p < 0.05, ‘**’ denotes p < 0.01, ‘***’ denotes p < 0.001, and ‘****’denotes p < 0.0001).

**ACCESSION NUMBERS**

aCGH data sets are available in the Gene Expression Omnibus database under the accession number GSE6676.

**SUPPLEMENTAL INFORMATION**

Supplemental Information includes Extended Experimental Procedures, six figures, four movies, two tables, and one data file and can be found with this article online at http://dx.doi.org/10.1016/j.cell.2014.04.042.

**AUTHOR CONTRIBUTIONS**

H.S., J.Z., and Y.C. supervised the experiments. H.S., T.W., and J.Z. designed the experiments. H.S. and T.W. performed confocal analysis, mtDNA copy number analysis, and mitochondrial replacement experiments. D.J., H.S., T.W., and H.L.Z. performed mtDNA genetic analysis. D.J., D.C., H.L.Z., and Y.C. performed aCGH analysis. T.W., H.S., and J.Z. prepared the figures and wrote the manuscript.

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